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Single point mutations in ATP synthase compensate for mitochondrial genome loss in trypanosomes

Samuel Dean^{1,2}, Matthew K. Gould¹, Caroline E. Dewar, and Achim C. Schnauffer³

Institute for Immunology and Infection Research and Centre for Immunity, Infection and Evolution, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3JT, United Kingdom

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Viability of the tsetse fly-transmitted African trypanosome *Trypanosoma brucei* depends on maintenance and expression of its kinetoplast (kDNA), the mitochondrial genome of this parasite and a putative target for veterinary and human antitrypanosomatid drugs. However, the closely related animal pathogens *T. evansi* and *T. equiperdum* are transmitted independently of tsetse flies and survive without a functional kinetoplast for reasons that have remained unclear. Here, we provide definitive evidence that single amino acid changes in the nuclearly encoded F_1F_0 -ATPase subunit γ can compensate for complete physical loss of kDNA in these parasites. Our results provide insight into the molecular mechanism of compensation for kDNA loss by showing F_0 -independent generation of the mitochondrial membrane potential with increased dependence on the ADP/ATP carrier. Our findings also suggest that, in the pathogenic bloodstream stage of *T. brucei*, the huge and energetically demanding apparatus required for kDNA maintenance and expression serves the production of a single F_1F_0 -ATPase subunit. These results have important implications for drug discovery and our understanding of the evolution of these parasites.

dourine | surra | dyskinetoplastic | RNA editing | mitochondrial DNA

Salivarian trypanosomes are extracellular protist parasites that cause important diseases in humans (human African trypanosomiasis) and their livestock (nagana). They predominantly infect the blood but, depending on the (sub)species, also other organs, such as the CNS. Transmission typically occurs through the saliva of blood-sucking insect vectors during feeding. The life cycle of African trypanosomes, such as *Trypanosoma congolense*, *T. brucei brucei*, *T. b. gambiense*, and *T. b. rhodesiense*, is fully dependent on cyclical development in the tsetse fly vector and highly complex (1). Thus, these parasites are restricted to areas inhabited by the tsetse fly (i.e., sub-Saharan Africa).

However, some pathogenic trypanosome species have adapted to efficient tsetse-independent transmission, abandoning any developmental stages associated with that vector, and therefore, they were able to escape from the African tsetse belt. *T. evansi* is transmitted mechanically by biting flies when the insect's blood meal on an infected host is interrupted and a second host is bitten with trypanosome-contaminated mouth parts shortly thereafter (2). *T. evansi* infects various mammalian animals, including livestock, and it is the pathogenic trypanosome with the widest geographical distribution. The disease caused by this parasite, therefore, has many different names but is known as surra in large parts of Asia. The second species, *T. equiperdum*, causes a sexually transmitted horse disease called dourine and predominantly infects genital tissues (2). *T. evansi* and *T. equiperdum* are morphologically indistinguishable from each other and *T. b. brucei*, and their status as independent species has been questioned (3, 4).

Mitochondrial DNA (mtDNA) of trypanosomatids is organized as the kinetoplast (kDNA), a gigantic network of concatenated, circular DNA molecules (5). The second key feature distinguishing *T. evansi* and *T. equiperdum* from *T. b. brucei* is that they are dyskinetoplastic [DK; i.e., lacking all (akinetoplastic or kDNA⁰) or critical parts (kDNA⁻) of their mitochondrial DNA] (6). *T. brucei* kDNA contains two types of molecules. The ~23-kb maxicircle, present in 20–50 copies, contains a typical set of rRNA and protein-coding genes, most of which encode subunits of

respiratory chain complexes (6). Most trypanosomatid mitochondrial mRNAs require a unique form of posttranscriptional editing before they can be translated into functional proteins (7). The second kDNA component is a highly diverse set of thousands of ~1-kb minicircles, which encode guide RNAs required for editing. Maintenance and expression of kDNA require numerous essential enzymes and have been suggested as a target for existing and novel drugs for *T. brucei* and other trypanosomatids (8). Indeed, antitrypanosomatid therapeutics, such as pentamidine and ethidium bromide, have been shown to directly affect kDNA (9, 10). *T. equiperdum* strains typically have retained their maxicircle—in some cases with substantial deletions—but have lost their minicircle diversity (4, 6). *T. evansi* strains do not have a maxicircle and either show minicircle homogeneity or are akinetoplastic. Consequently, both species are incapable of functional mitochondrial gene expression.

T. evansi and *T. equiperdum* cannot develop in the tsetse fly, probably because ATP production in that environment requires oxidative phosphorylation (11) and, therefore, the capacity to express numerous mitochondrial genes. They can only survive as bloodstream forms (BFs), which produce ATP exclusively through glycolysis; however, they still require a mitochondrion, because it hosts other essential activities (12–14). A key process underpinning mitochondrial function is the maintenance of an electrochemical potential, $\Delta\psi_m$, across the inner mitochondrial membrane (15). BF *T. brucei*, which lacks the proton pumping respiratory complexes III and IV, generates $\Delta\psi_m$ using the mitochondrial F_1F_0 -ATP synthase complex functioning in reverse to pump protons from the matrix into the intermembrane space (16–18). Subunit a of the membrane-embedded F_0 part is critical for proton translocation (Fig. S1A) and kDNA-encoded, and its pre-mRNA requires substantial RNA editing (19). DK trypanosomes are incapable of expressing subunit a , because they lack either the gene itself or most, if not all, guide RNAs. The puzzling fact that these organisms are viable was hypothesized to involve compensatory mutations in the nuclearly encoded F_1 subunit γ (Fig. S1B–D) that enable F_0 -independent $\Delta\psi_m$ generation (4, 17).

We tested this hypothesis by generating BF *T. b. brucei* that express mutated γ subunits and investigating their response to kDNA loss. Our results show that a single amino acid change in subunit γ fully compensates for complete loss of kDNA and provide insight into the molecular mechanism of this compensation. This finding has important consequences for our understanding of

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¹S.D. and M.K.G. contributed equally to this work.

²Present address: Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, United Kingdom.

³To whom correspondence should be addressed. E-mail: achim.schnauffer@ed.ac.uk.

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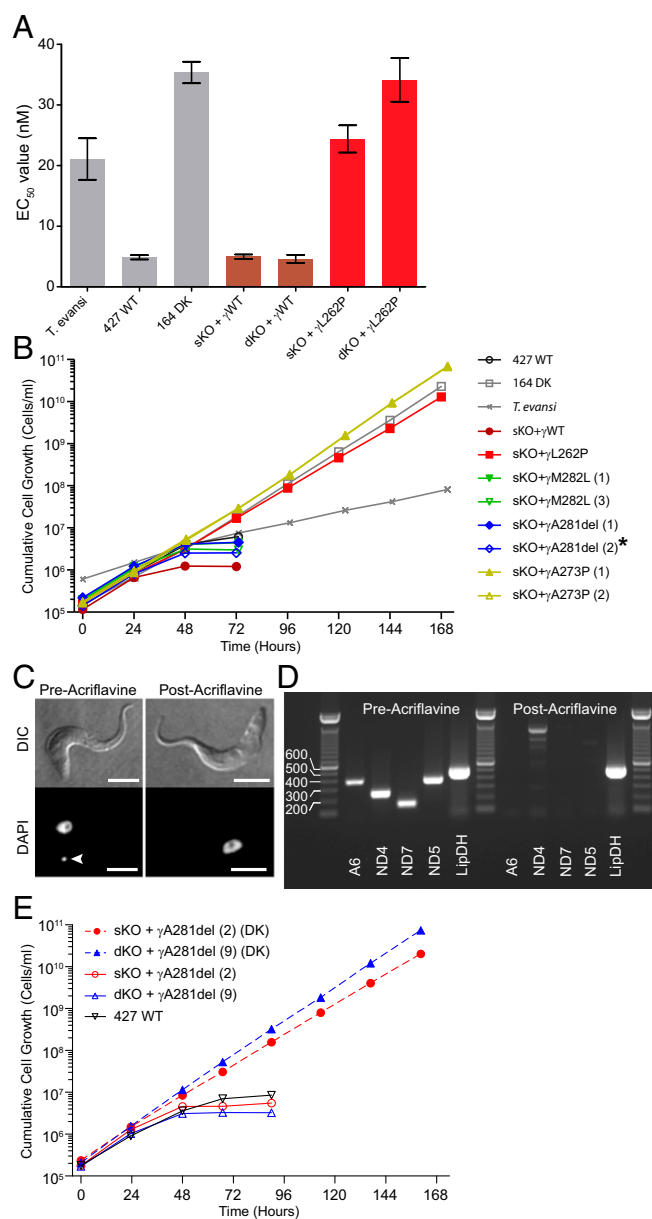


Fig. 1. Mutations in ATPase γ allow BF *T. b. brucei* to survive kDNA loss. (A) Acr sensitivity of γ L262P-expressing and control BF trypanosomes given as EC₅₀ values. Error bars are SEM; $n \geq 3$. (B) Cumulative growth in 20 nM Acr of cells ectopically expressing WT F₁₇ or an L262P, A273P, A281del, or M282 mutated copy in an sKO background. Numbers indicate independent clones. Parental WT strain 427, *T. b. brucei* 164DK, and *T. evansi* Antat 3/3 were also analyzed. [Fig. S2 A and B](#) shows growth curves without Acr and for dKO cells. (C) Differential interference contrast (DIC) and fluorescence microscopy of DAPI-stained dKO + γ L262P trypanosomes before and after exposure to 20 nM Acr. White arrowhead in pre-Acr exposure images indicate the kinetoplast. (Scale bars: 5 μ m.) (D) PCR assay for presence of kDNA-encoded genes A6, ND4, ND7, and ND5 in dKO + γ L262P cells before and after Acr exposure. The faint band observed with ND4 primers post-Acr treatment is a result of nonspecific amplification, which is shown by its larger size. The nuclearly encoded dihydroliipoamide dehydrogenase gene (*LipDH*) was assayed as a positive control. (E) Cumulative growth in the presence of 20 nM Acr of previously Acr-treated (and therefore, DK) γ A281del clones 2 and 9 after they had been allowed to recover in Acr-free medium (dashed lines; B and [Fig. S2B](#) show the initial response of these clones to Acr exposure). The same clones but without any prior Acr exposure were included in the analysis (solid lines). The parental *T. b. brucei* 427 strain was assayed for comparison.

These results suggested that strains of *T. evansi* and *T. equiperdum* with an A281del or A273P mutation depend on these mutations to be viable. To test this suggestion further, we inducibly expressed either γ WT or γ L262P in *T. evansi* Antat 3/3 (these parasites are heterozygous for the A281del mutation; see Table 1). Expression of γ WT caused a strong growth defect after 48 h, whereas expression of γ L262P had no effect (Fig. 2A), suggesting that replacement of sufficient endogenous (A281del-mutant) γ subunits in the ATP synthase complex with WT subunits severely impairs the viability of these DK cells. This observation also suggested that the L262P mutation from the laboratory-induced DK strain of *T. b. brucei* and the A281del mutation present in *T. evansi* Antat 3/3 are, at least to some extent, functionally interchangeable. In another experiment, we induced expression of an ectopic γ L262P allele in *T. b. brucei* 427 cells and then treated the cells with 20 nM Acr. As predicted, these cells were resistant to Acr and rapidly lost their kinetoplast. Subsequent repression of the γ L262P allele forced these DK cells to rely on endogenous γ WT alone and caused a severe growth defect after 24 h (Fig. 2B). In contrast, the growth rate of cells with maintained expression of γ L262P remained constant. These results confirm that, after expression of a mutated F₁ γ subunit has permitted loss of kDNA, the DK trypanosomes now depend on continued expression of this mutated subunit to remain viable.

Because specific $F_{1\gamma}$ mutations are able to compensate for kDNA loss, all genes exclusively involved in kDNA biogenesis or expression would be predicted to become dispensable. RNA editing ligase 1 (REL1) is essential in the kinetoplast mRNA editing process and its knockdown lethal (23). To determine whether the L262P mutation can compensate for REL1 loss, the γ L262P gene or the γ WT control was constitutively expressed in *T. b. brucei* 427 engineered for inducible REL1 RNAi. Contrasting with γ WT cells, γ L262P-expressing trypanosomes showed no growth effect after RNAi-mediated knockdown of REL1 (Fig. 3). Interestingly, γ L262P-expressing *T. brucei* was not viable after ablation of mitochondrial topoisomerase II (*TbTop2mt*), a protein essential for kDNA replication (24). To validate this observation, we ablated *TbTop2mt* expression in *T. evansi* Antat 3/3 and observed a growth defect after 48 h (Fig. S5A). Secondary effects resulting from faulty kDNA replication (for example, potentially toxic accumulation of kDNA) are unlikely to be responsible, because the Antat 3/3 strain used for this study is kDNA⁰ (Fig. S5B) (17), probably as a result of long-term in vitro culture (25). The most likely explanation for the growth defect in *T. evansi* is, therefore, that *TbTop2mt* has an additional important function outside of its role in kDNA replication. Nevertheless, together with the experiments investigating chemically induced kDNA loss, these genetic data show that specific point mutations of ATPase γ are fully sufficient to compensate for loss of kDNA or its gene expression.

The proton-translocating function of the membrane-embedded F_0 part of the ATP synthase involves subunit a , which is thought to be the only ATPase subunit encoded in the kinetoplast (Fig. S14). To test directly whether a mutated ATPase γ is necessary for generating $\Delta\Psi_m$ in kDNA⁰ cells, we measured $\Delta\Psi_m$ in BF trypanosomes over the course of Acr treatment. For cells expressing only γ WT, $\Delta\Psi_m$ decreased after 24 h and was completely abolished after 48 h (Fig. 4A), preceding cell death by at least 24 h. In contrast, $\Delta\Psi_m$ of the γ L262P-expressing trypanosomes was not affected by Acr-induced kDNA loss (Fig. 4B). The slight decrease in $\Delta\Psi_m$ during Acr exposure is probably the result of kDNA-independent Acr toxicity, because no difference in $\Delta\Psi_m$ could be observed after removal of Acr from the medium (Fig. S6). The current model for $\Delta\Psi_m$ generation in DK trypanosomes proposes that increased ATP hydrolysis by a mutated F_1 part facilitates the electrogenic exchange of cytosolic ATP⁴⁻ for mitochondrial ATP³⁻ by the ADP/ATP carrier (AAC) (17, 26). We measured sensitivity of our cell lines to oligomycin, an inhibitor of the coupled F_1F_0 -ATPase, and the AAC inhibitor bongkreikic acid. Trypanosomes expressing mutant ATPase γ showed similar levels of oligomycin resistance before and after Acr-induced kDNA loss

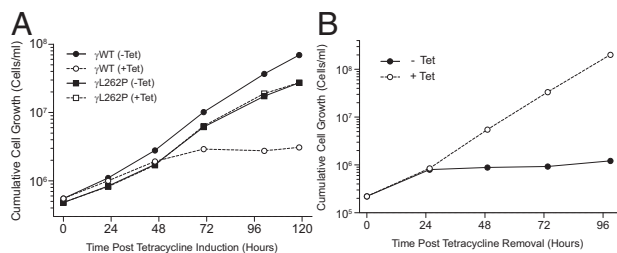


Fig. 2. Viability of DK trypanosomes depends on expression of a mutated $F_1\gamma$. (A) Cumulative growth of *T. evansi* with Tetracycline (Tet) -inducible ectopic expression of a WT (circles) or L262P-mutated (squares) subunit γ (dashed lines and open symbols, + Tet; solid lines and closed symbols, -Tet). (B) A Tet-inducible γ L262P was expressed in *T. b. brucei* cells, and kDNA loss was triggered by exposure to 20 nM Acr for 7 d. The culture was split, and expression of γ L262P in one subculture was repressed by transfer to Tet-free medium (0 h). Cumulative cell growth in the presence (dashed line and open circles) or absence (solid line and closed circles) of Tet was determined.

(Fig. 4C), suggesting that these mutations fully uncouple the F_1 and F_O activities, even in kDNA⁺ cells. In support of the proposed importance of the AAC for $\Delta\Psi_m$ generation in the absence of a proton-pumping F_OF_1 -ATPase, cells expressing γ L262P showed increased sensitivity to bongkreik acid. Again, this change in sensitivity was irrespective of the presence or absence of kDNA (Fig. 4D), sustaining the hypothesis that, even in the presence of a functional F_O , the mutation results in a complete switch to the alternative mode of $\Delta\Psi_m$ generation.

Discussion

The apparently conflicting observations that BF *T. brucei* depends on kDNA for survival (23, 27, 28), whereas the closely related species *T. evansi* and *T. equiperdum* as well as certain laboratory-generated *T. brucei* strains are viable, despite the loss of all or critical parts of their kDNA (6), have generated a puzzling conundrum. In this report, we show that specific single amino acid mutations in the nuclearly encoded γ subunit of the mitochondrial ATP synthase complex are fully sufficient to compensate for loss of kDNA-encoded gene products in the BF parasite. The underlying biochemical mechanism involves uncoupling of the F_1 and F_O parts of the enzyme and increased dependence on a highly active AAC. Some field isolates seem to depend on additional adaptations that remain to be identified.

Four different candidate mutations have been identified to date (Table 1). We found that the L262P and A273P mutations are fully sufficient to permit normal growth of BF parasites in the absence of kDNA. Neither chemically induced loss of the kinetoplast nor loss of kDNA expression by RNAi resulted in any lag in growth that would have indicated the requirement for additional adaptations. This finding also rules out a potential kDNA replication checkpoint for cell cycle completion (29). More than 40 y

after the generation of the only surviving DK *T. b. brucei* strain (20), its viability can now be explained. Likewise, the A273P mutation present in certain field isolates of *T. equiperdum* strains is fully sufficient to explain why these strains are viable, despite the loss of almost all minicircle classes or the entire kDNA (Table 1) (4). Despite the fact that the γ -mutation in these field isolates is homozygous, presence of a single WT allele did not significantly affect survival during or after kDNA loss. Thus these mutations can be classified as dominant, although the relative expression levels of WT and mutant allele in our experimental strains and, therefore, the degree of dominance, are uncertain. The effect of the A281del mutation was less robust: several independent clones either survived kDNA loss only after a significant lag in growth, indicative of a requirement for secondary adaptations, or did not survive at all. The nature of this additional adaptation remains to be determined. Finally, the M282L mutation did not increase the parasite's ability to tolerate kDNA loss. It could still be part of the mechanism that allows this particular *T. evansi* strain to survive but by itself, be insufficient, or it could be of no significance. The strain in question is a Kenyan isolate and representative of the rare type B strains (30) (Table 1).

What is the biochemical mechanism that enables BF trypanosomes to generate a $\Delta\Psi_m$ in the absence of a functional F_O part? Our study shows that introduction of an $F_1\gamma$ L262P mutation results in oligomycin resistance and, consequently provides direct evidence that it uncouples the F_1 and F_O parts of the enzyme. This scenario is reminiscent of *mg1* mutations in yeast (Fig. S1 B–D), although in that organism, it is not clear whether uncoupling is directly linked to viability without a mitochondrial genome (31). A reduced K_m for ATP was also suggested as part of the mechanism for yeast *mg1* mutations (32), and the effects of the trypanosome γ mutations on F_1 -ATPase kinetics remain to be investigated. Both mechanisms could result in increased ATPase activity and be necessary to provide sufficient ADP^{3-} for an efficient electrogenic exchange with ATP^{4-} by the AAC (17, 26, 32). Our finding of bongkreik acid hypersensitivity in cells expressing the mutant γ shows an increased importance for the role of the AAC in these cells. There is evidence in yeast and *Leishmania* for an AAC/ F_1F_O -ATPase supercomplex (33, 34), which might explain the apparent preference for the ATPase as the source of ADP^{3-} in mtDNA⁰ cells. Such a supercomplex might generate localized zones of increased $\Delta\Psi_m$ and, in association with the protein import machinery, exploit them for protein import, similar to the supercomplex of Tim21 and respiratory complexes III and IV reported for yeast (35). A number of protists, including many important parasites, lack classical mitochondria and instead, harbor related organelles classified as hydrogenosomes or mitosomes that usually lack an organellar genome. How these organelles generate a $\Delta\Psi_m$ is unclear (36, 37), and trypanosomes promise to be an excellent experimental system to further investigate this important problem in cell biology.

How did *T. evansi* and *T. equiperdum* evolve from *T. brucei*, and can the findings reported here help to address this question?

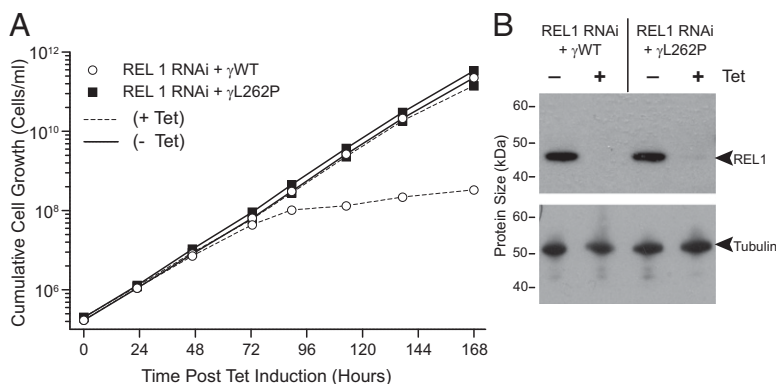


Fig. 3. The L262P mutation allows BF *T. b. brucei* to survive inhibition of mitochondrial gene expression. (A) Cumulative growth of *T. b. brucei* after RNAi-mediated knockdown of REL1 (dashed lines). Cells expressed either γ WT (open circles) or γ L262P (closed squares). RNAi was induced with 1 μ g/mL Tet; uninduced control cultures are shown as solid lines. (B) Western analysis of REL1 protein expression from whole-cell lysates taken at the 72-h time point in A; α -tubulin was used as a loading control (Lower).

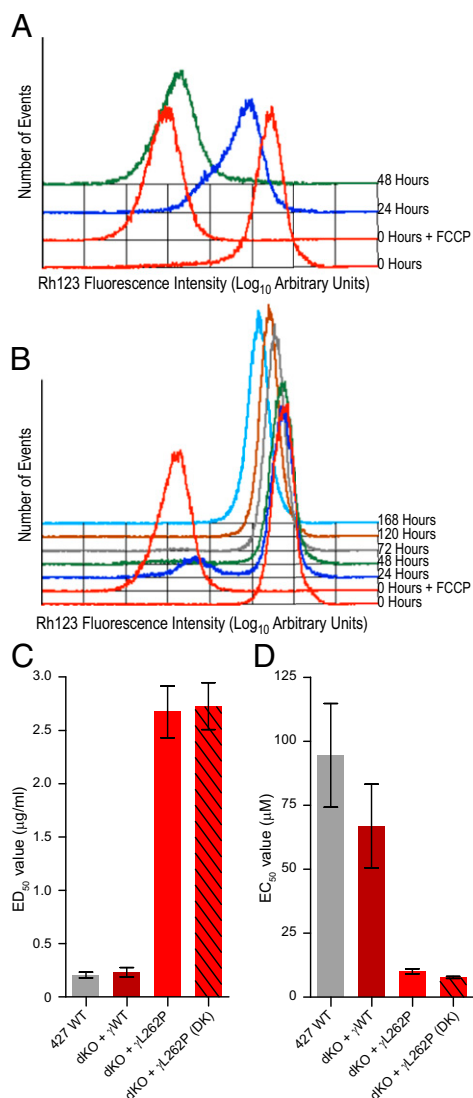


Fig. 4. Subunit γ mutations that can compensate for kDNA loss in BF *T. b. brucei* uncouple F_1 and F_0 and prevent $\Delta\Psi_m$ loss. (A and B) $\Delta\Psi_m$ of BF cells continuously cultured with 20 nM Acr and expressing either ectopic (A) γ WT or (B) γ L262P in a γ dKO background. $\Delta\Psi_m$ was assessed using rhodamine 123 and flow cytometry. Baseline fluorescence was determined by preincubation with the protonophore trifluorocarbonyl cyanide phenylhydrazide (FCCP) (0 h + FCCP). In A, insufficient cells survived beyond 48 h to accurately determine $\Delta\Psi_m$. (C) Oligomycin sensitivity (ED_{50} values) of trypanosomes expressing either γ WT or γ L262P in a γ dKO background. The Acr-induced DK form of the γ L262P-expressing cell line was assayed in parallel along with the parental WT 427 strain. Error bars are SEM; $n \geq 3$. (D) The same as in C, except assessing bongkrekic acid sensitivity (given as EC_{50} values).

The correlation between loss of a functional kinetoplast and an oligomycin-sensitive F_1F_0 -ATPase on the one hand and loss of fly transmissibility on the other hand was first recognized by Opperdoes et al. (38). The temporal order of the two critical events in the evolutionary history of these parasites—acquisition of a propensity for efficient tsetse-independent transmission (the basis of which is still mysterious) and occurrence of compensatory changes that allowed kDNA loss—has been debated elsewhere (39–42). However, regardless of whether the γ subunit mutation occurred before or after adaptation to nontsetse transmission, it is evident that it was a key event and, thus a valuable marker for tracing that history. Each mutation in Table 1 is specifically correlated with other genetic polymorphisms (42), and it is, therefore,

likely that isolates sharing the same mutation also share the same ancestor. Importantly, the largest group, characterized by the A281del mutation, contains isolates from Africa, Asia, and South America collected during a span of 100 y, and it contains both *T. evansi* and *T. equiperdum* isolates. Either these *T. equiperdum* isolates are *T. evansi* isolates that had been misclassified (3) or many isolates of these two species are descendants of the same evolutionary event (43, 44). Based on these considerations, we would propose that, contrary to what was proposed recently (4), the extant strains seem to be the result of a limited rather than large number of independent evolutionary events. It has been suggested that *T. evansi*/*T. equiperdum* are analogous to yeast petite mutants (4, 38), and, indeed, the mechanism that allows mtDNA loss in petite-negative yeast and *T. brucei* is strikingly similar (17). However, it is important to stress that the ability to survive without a mitochondrial genome is only one distinctive feature of these parasites, because the other one, efficient tsetse-independent transmission, has had such dramatic epidemiological consequences.

Maintenance and expression of kDNA has been suggested as a target for existing and novel antitrypanosomatid drugs (8–10). In fact, the Acr compound used for kDNA elimination in the present study was originally developed with the aim of finding a cure for sleeping sickness (45). The fact that single nucleotide changes can make the parasite completely independent of kDNA and its expression suggests that this target needs to be treated with caution, at least for *T. brucei*. However, a few considerations are important. First, an uncoupled F_1F_0 enzyme will be incapable of proton gradient-driven ATP synthesis, which is thought to be required for survival in the tsetse vector because of the low abundance of glucose in the insect's midgut (11, 46). The mutation would, consequently be expected to lock the parasite in the mammalian host and prevent spread of resistant parasites. Second, other pathogenic trypanosomatids, including *T. congolense*, *T. cruzi*, and *Leishmania* spp., do not seem to be able to circumvent the need for functional kDNA, which therefore, remains a highly promising target in those parasites.

Another important conclusion from our study is that ATP synthase subunit *a* seems to be the sole kDNA product ultimately required for viability of BF *T. brucei*. A subunit of the mitochondrial ribosome, RPS12, may be encoded in kDNA (47), and it has been reported that the product of an alternatively edited mRNA, *AEP-1*, is required for kDNA maintenance (48); however, these proteins would also become dispensable, along with subunit *a*, in the presence of a compensatory ATP synthase γ -mutation. The same is true for the numerous nuclearly encoded proteins required exclusively for maintenance and expression of kDNA (5, 7, 49). Thus, a single amino acid mutation in BF *T. brucei* makes a large number of otherwise essential proteins redundant. The compensatory mutations reported in this paper offer an attractive tool for their identification and characterization.

Materials and Methods

Materials, Trypanosome Strains, and Culture Conditions. Details on materials can be found in *SI Materials and Methods*. All experimental work and culturing were carried out with BF trypanosomes only. ATP synthase γ (Tb927.10.180) genetic manipulations in *T. b. brucei* were conducted on the Lister 427 strain, except for inducible expression and RNAi experiments, which used the 427 single marker cell line (50). Inducible expression in *T. evansi* was conducted in a cell line expressing T7RNAP and TETR (17). Cell lines *T. evansi* Antat 3/3 (51) and *T. b. brucei* DK 164 (20) were included in growth experiments for comparison. *SI Materials and Methods* has details on culturing, plasmid construction, and transfection. See *Table S2* for oligonucleotides.

Alamar Blue Dose–Response Assay. The Alamar Blue assay was performed essentially as described (21) with minor modifications. Briefly, test compound was doubly diluted in 100 μ L Hirumi-modified Iscove's medium 9/10% (vol/vol) FBS in a 96-well plate; an equal volume of medium containing BF trypanosomes was added to give a final density of 5×10^3 cells/mL. The plate was incubated at 37 °C and 5% (vol/vol) CO₂ for 72 h, after which 20 μ L 0.5 mM resazurin sodium salt in PBS were added to each well; then, the plate was incubated for another 4 h. Fluorescence was measured in a plate reader with excitation and emission filters of 544 and 590 nm, respectively. EC₅₀/

ED₅₀ values were derived from a variable slope (four parameter) nonlinear regression using Prism 5 software (GraphPad).

DAPI Staining. Trypanosomes were washed in PBS, settled onto poly-L-lysine coated slides, and fixed in 3% (wt/vol) formaldehyde for 10 min before treating with excess cold methanol for at least 30 min. Slides were then rehydrated in PBS and mounted in glycerol containing 1 µg/mL DAPI before imaging using a Leica SP5W5C2 confocal laser microscope (blue diode laser at 405-nm wavelength) with Velocity version 5.2 image analysis software (PerkinElmer).

PCR and Western Analyses. Details are given in *SI Materials and Methods*.

ΔΨm Measurements. Samples of trypanosome cultures, either exposed or unexposed to Acr, were incubated with 260 nM rhodamine 123 (Rh123) for

20 min at 37 °C. Cells were harvested by centrifugation at 1,300 × g for 10 min and washed one time with 25 mM Hepes, pH 7.6, 120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄/KH₂PO₄, pH 7.6, 2 mM EDTA, 5 mM MgCl₂, and 6 mM D-Glucose. Fluorescence caused by Rh123 uptake was measured using a FACSCalibur flow cytometer with CellQuest Pro software (Becton Dickinson). Baseline fluorescence was established for each sample by preincubating an aliquot of cells with 10 µM FCCP before adding Rh123; the FCCP concentration was maintained throughout the wash and flow cytometer steps.

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